American-Eurasian Journal of Scientific Research 2 (2): 170-179, 2007
ISSN 1818-6785

Enhancement of Antioxidant Production in *Spirulina Plantensis* Under Oxidative Stress

[1]Hanaa H. Abd El-Baky, [1]F.K. El Baz and [2]Gamal S. El-Baroty

[1]Department of Plant Biochemistry, National Research Centre, Dokki, Cairo, Egypt
[2] Department of Biochemistry, Faculty of Agriculture, Cairo University, Egypt

Abstract: The present study examines the possibility of increasing the levels of some bioactive compounds in *Spirulina plantensis* cultivated in media containing various hydrogen peroxide concentrations (2, 4, 6 and 8 mM) as a model for environmental stress. *Spirulina plantensis* showed significant linear increase in antioxidant enzyme activities, i.e., catalase (CAT), peroxidase (PX), Ascorbate Peroxidase (AXP) and superoxide dismutase (SOD), with increasing H_2O_2 levels. A pronounced decrease of oxidative lesions indexes (thiobarbituric acid reactive substances (TBARs) and alkyl radical-PER signal) was noticed. HPLC profile of carotenoids and tocopherols of treated algae revealed that algae responded to the change of H_2O_2 exposure by modifying their cellular contents and compositions. Significant positive correlation was observed between the increase of H_2O_2 in media and increasing the amounts of astaxanthine, lutein and α-tocopherol. Also, hydrophilic antioxidants (glutathione and ascorbic acid content) were increased with increasing H_2O_2 concentration. These data revealed that *Spirulina plantensis* behaved differently strategies against H_2O_2 exposure which is dose dependent and strongly correlate with the activities of antioxidant enzymes and antioxidant compounds. Therefore, make *Spirulina plantensis* good candidates for successful cultivation in artificial open ponds under different environmental conditions as high value health foods, functional foods and as source of wide spectrum of nutrients.

Key words: *Spirulina plantensis* . carotenoids . antioxidant enzymes . ascorbic acids . tocopherols and free radicals

INTRODUCTION

Hydrogen peroxide is a product of microalgae and plants through of photosynthesis, photorespiration, respiration and other metabolic processes, as result from the enzymatic activity of glycolate oxidase, urate oxidase and amino acid oxidase [1]. However, major pathway for production of H_2O_2 is conversion from superoxide (O_2^-) produced through the transfer of an electron from ferredoxin of photosystem I (PSI) to O_2 (Mehler reaction) by the action of Superoxide Dismutase (SOD). Also, SOD produces H_2O_2 from other superoxide sources such as mitochondria SOD [2], cytochrom P450 system and enzymatic activity of xanthine oxidase and galactose oxidase [1, 3]. However, H_2O_2 and other reactive oxygen species (ROS) produced in a number of cellular reactions, including the iron-catalysed Fenton reaction and by various enzymes such as lipoxygenases, peroxidases and NADPH oxidase [4]. H_2O_2 and other ROS in a constantly produced in all aerobically living cells and these are potentially toxic to cells [5]. For example, H_2O_2 can injure cells at the higher concentration or lead to acclimation at moderate levels [6]. H_2O_2 itself is not particularly react with most biologically important molecules but it is probably an intracellular precursor for more reactive oxidants as it passes quickly through membranes by diffusion [7]. Consequently, reduces photosynthesis process by inhibiting a number of photosynthetic enzymes [3], such as fructose 1,6 bisphosphatase, ribulosesphosphate kinase and ribulose bisphosphate carboxlase, oxygenase and other enzyme such as SOD [1, 8. The uncontrolled production of ROS molecules by protective system, may destroy proteins, lipids and pigments. The xanthophylls cycle in the thylakoid membranes dissipates excess energy and help plants to avoid the production of singlet oxygen [9].

Algae contain several enzymatic and non-enzymatic antioxidant defense systems to maintain the concentration of ROS (O_2^- and H_2O_2) to protect cells from damage [10-12]. The main cellular components susceptible to damage by these ROS are lipids (peroxidation of poly-unsaturated fatty acids in

Corresponding Author: Dr. Hanaa H. Abd El-Baky, Department of Plant Biochemistry, National Research Centre, Dokki, Cairo, Egypt

membranes), proteins (denaturation), carbohydrates and nucleic acids [13, 14]. The essential for ROS detoxification during normal metabolism and particularly during stress, are antioxidant defenses system [14]. The primary scavenging enzymatic defenses system include SOD, calalase (CAT) and glutathione peroxidase, (GPX) and peroxiredoxin (PrxR) [11, 15]. These enzymic detoxification system involving the action of SOD and reductase, either quench toxic compounds or regenerate antioxidants with the help of reducing power provided by photosynthesis [16]. Non-enzymatic defenses include carotenoids, tecopherols (TOH)) and ascorbic acid (AA), glutathione (GSH) and chlorophyll derivates. Both systems provide adequate protection against effects ROS, under stress condition [17]. The changes in enzyme and non-enzymatic system have been the focus of several algae stress studies. It was postulated that increases in antioxidant levels are correlated with reduce the cells injury [18-20]. One the contrary, decreased antioxidant activity in stressed tissue resulted in higher levels of AOS may lead to injury [21]. However, at low levels, H_2O_2 resulted in induction of defense genes such as glutathione S-transferase and glutathione peroxidase. The hydrophilic antioxidants AA and GSH effectively scavenge oxygen radicals. Carotenoids and TOH remove ROS directly from the pigment bed [22]. Finally, Foyer and Noctor [23] reported that the changes in ROS, fluctuations in the antioxidants concentrations in photosynthetic cells might have important consequences not only for defense metabolism but also for the regulation of genes associated with adaptive responses.

The aim of the present study was performed to determine the response of *Spirulina plantensis* to H_2O_2 stress, which might be used as a possible source for antioxidative substances for commercial or pharmaceutical purpose.

MATERIALS AND METHODS

Algae source: *Spirulina plantensis* was obtained from the Culture Collection of Texas University, Austin, Texas, USA.

Growth conditions: Alga was cultured in 4 L Erlenmeyer flasks containing autoclaved 3 L Zarrouk's medium [24]. The medium was enriched with H_2O_2 (Sigma, 30%, w/v) at source in concentrations of 0, 2, 4, 6 and 8 mM. The pH of the medium was adjusted to pH 10.5 with 1 M NaOH prior to autoclaving. The cultures were gassed with 0.3% CO_2 in air and the algae were cultivated at 25ºC±3. The cultivated flasks were illuminated 24h with continuous cool white fluorescent lamps at 400 W (Philips).

Growth measurements: The growth of *Spirulina plantensis* was spectrophotometrical measured according to Payer [25] methods.

Harvesting: The algal growth culture at stationary-phase was harvested by centrifugation at 6,000 X g for 15 min at 4°C and frozen at-20°C.

Extraction of lipophilic antioxidants: Carotenoids and tocopherols were extracted from algal cells with tetrahydrofuran (1: 10 w/v) in the present of BHT (30 mg g^{-1}) (2,6 ditertra-butyl-p-cresol) and magnesium carbonate (100 mg g^{-1}). After 24 h, an aliquot of the clear extracted pigments was filtered and evaporated to sample volume of 5 mL under a stream of nitrogen. The extracted pigments were saponified with methanolic potassium hydroxide (25 mL of 10%) for 2 h at room temperature, then the lipophilic antioxidants were extracted with dichloromethane. The solvent layer was then separated, washed several time with distilled water, then dried over Na_2SO_4 and dried under stress of nitrogen.

Determination of algal total carotenoids: Total carotenoids were determined by spectrophotometrically method at 450 nm and β-carotene served as a standard compound was used for preparing the calibration curve [26].

Characterization of carotenoids by HPLC: Separation and identification of carotenoids were carried out by Liquid hromatographic system. The system consisted of a spectra system UV 2000 detector (hold at 438 nm), spectra system P2000 pump and Chromosil C_{18} analytical column (25 cm x 4.6 mm i.d, 5 µm particle size). Mobile phase was 8:1 (v/v) acetonitrile: methanol, at flow rate of 1 ml min^{-1}. Samples were dissolved in mobile phase and 10 µl was injected. Some available standard carotenoids: β-carotene, zeaxanthin, lutein, astaxanthin and cryptoxanthin (Sigma) were also run by the same HPLC method [20, 27].

Determination of algal tocopherols: Tocopherols were determined by HPLC method. HPLC system equipped with Spectra System UV2000 detector at 290 nm and separated on Vydac analytical column (25 cm X 4.6 nm i.d, 5 µm particle size). Tocopherols were eluted with acetonitrile: methanol (9:1 v/v) at a flow rate of 1 ml min^{-1}. Standard of α-tocopherol with purity not lass than 99% (Sigma) was run under the same conditions [20].

Extraction and determination of ascorbic acid: Ascorbic acid (vitamin C) was extracted from the

algal cells with 2% metaphosphoric acid and spectrophotometrically determined by using 2, 6 di-chlorophenol indophenol dye [28].

Preparation of cytosolic fraction: Algae cells were excised and homogenized with ice cold extraction buffer (5ml 250 mM sucrose and 25 mM Tris, pH 7.2). The homogenate was centrifuged at 16,000 Xg for 20 min at 4°C. The supernatant was used for the estimation of glutathione (GSH), lipid peroxidation, protein content and enzyme assay.

Enzymes assays: The activities of cytosolic SOD (EC, 1.15.1.1) and PX activity (EC, 1.11.1.7) were spectrophotometrically [29, 30] respectively. The CAT enzyme (EC, 1.11.1.6) activity was assayed spectrophotometrically by the decomposition of H_2O_2 at 240 nm in a reaction mixture containing of a 10 mM H_2O_2 and 25 mM phosphate buffer, pH 7.0 [31]. APX (EC 1.11.1.11) was determined according to Nakano and Asada [32].

Determination of glutathione (GSH): The GSH content of algal cell extracts was measured by reaction with 5,5` dithiobis-2-nitrobenzoic (DTNB) according to Silber *et al*. [33]. The level of GSH was expressed as µM.

Determination of lipid peroxidation products: The lipid peroxidation products in algal cell extracts were estimated by determining the thiobarbaturic acid reactive substances (TBARS) as described by Haraguchi *et al*. [34]. The lipid peroxidation was expressed as micromoles of malonaldhyde (MDA).

Detection of free radicals by ESR-Spain: The algal cells (0.2 g) from both control and treatment were lyophilized in a speed Vac Sc 100 (Savant). The powdered cells (0.05 g) were mixed with 1,1 diphenyl-2 picrylhydrazyl (DPPH). No increased in back ground ESR signal occurred when DPPH alone was exposed to UV-light. ESR spectra were obtained at room temperature (approx. 18°C) using a Bruker (Karlsruhe, Germany), Spectrometer ELEXSYS E-500 was operated at 9.808 GHz with 100 kHz modulation frequency. EPR instruments settings for the Spin trapping experiments were: microwave power, 20.2 mW; modulation amplitude 1.0 G; time constant, 51 ms; receiver gain 60.0. EPR for all samples were recorded at exactly the same spectrometric settings and the first derivative EPR spectra were double-integrated to obtain the area intensity.

Determination of proteins: Protein content of algal buffer extracts was estimated according to Bradford [35], at 595 nm, using comassein blue G 250 as a protein binding dye. Bovine serum albumin (BSA) was used as a protein standard. Protein concentrations in the samples were calculated from the calibration curve, in mg protein in ml extract. Activities of SOD, CAT and POD were normalized to protein concentration.

Statistical analysis: The experiments were done in triplicate. Data are expressed as the means ±SD (standard deviation). Results were analyzed by one-way ANOVA and Student's t-test at significance level of 5%. All analyses performed using Co Stat software version 4 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS AND DISCUSSION

Effect of different H_2O_2 concentrations on *Sp. plantenes* growth: The addition of H_2O_2 at different concentrations (2, 4, 6 and 8 mM) to culture media of *Sp. plantenes* was regarded as indication for tolerance against artificial H_2O_2 stress (Fig. 1). The dry biomass of algal (expressed as mg L^{-1} DW) was gradually reduced under H_2O_2 stress in dependency of exposure time and H_2O_2 concentration. After third and sixth days of exposure, a dramatic decrease in biomass was recorded at 2-8 mM H_2O_2. The dry biomass value was significantly reduced from 70 to 52% of the control (100%). After 9, 12, 15 and 18 days of exposure, the D.W values was about 84, 82 81 and 79%, respectively of the control in cells grown at 2 mM of H_2O_2. While, after 21 and 24 days, the DW was reduced and reached to about 92 and 96%, respectively of the control. No bleaching process was noticed at these levels concomitantly the cells exhibited much softer consistence. Under the highest H_2O_2 level, DW value was significantly reduced to 52, 51, 56, 77, 68, 68, 72 and 72% of the control (100%), after 3, 6, 12, 15, 18 and 21 days respectively and the cells did not start to bleach. In general, the results revealed that *Sp. plantenes* tolerated up to 8 mM H_2O_2, which the biomass was still

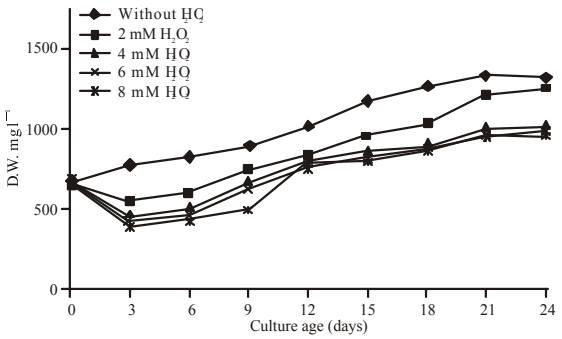

Fig. 1: Effect of hydrogen peroxide on growth (Dry weight mg l^{-1}) of *Spirulina plantensis*

Table 1: Effect of hydrogen peroxide on antioxidant substances of *Spirulina plantensis*

	α-Tecopherol		Ascorbic acid		GSH	
Treatment	mg g^{-1}	Ratio	mg g^{-1}	Ratio	µM	Ratio
Control without H_2O_2	0.96	1.00	3.80	1.00	88.32	1.00
2 mM H_2O_2/L	1.69	1.76	7.11	1.87	115.2	1.30
4 mM H_2O_2 /L	4.31	4.48	9.39	2.47	163.7	1.80
6 mMH_2O_2/L	5.61	5.84	11.23	2.95	190.8	2.16
8 mM H_2O_2/L	8.36	8.71	16.35	4.30	223.6	2.50

Table 2: Influence of hydrogen peroxide on caroteniods of *Spirulina plantensis*

	β-carotene		Astaxanthine		Luteine		Zeaxanthine		Cryptoxanthine		Total carotenoids		
Treatment	µg g^{-1}	Ratio	µg g^{-1}	Ratio	µg g^{-1}	Ratio	µg g^{-1}	Ratio	µg g^{-1}	Ratio	mg g^{-1}	%	Ratio
Control	39.12	1.00	5.61	1.00	0.301	1.00	1.56	1.00	1.69	1.00	4.61	0.46	1.00
2 mMH_2O_2	62.35	1.59	9.11	1.62	0.510	1.70	2.65	1.70	2.35	1.40	8.36	0.83	1.81
4mM H_2O_2	90.35	2.31	14.21	2.53	0.981	3.26	3.21	2.06	3.65	2.16	15.11	1.51	3.28
6 mMH_2O_2	124.32	3.18	18.13	3.23	1.180	3.92	3.65	2.34	4.98	2.95	22.21	2.22	4.80
8mM H_2O_2	133.54	3.41	26.14	4.66	1.320	4.38	5.66	3.63	6.69	3.96	35.25	3.52	7.65

All values are significant at (P<0.5)

about 50% of the control culture and the cells did not start to bleach this mean that the photosynthetic activity was found of H_2O_2, during the entering experiment. Same pattern was observed thought out the experimental periods for other concentration levels.

Effect of oxidative stress on antioxidant compounds:

In order to determine the compounds that may influence the resistance of *Sp. plantensis* to H_2O_2 stress, the algae cells were extracted with lipophilic and hydrophilic solvents. Subsequently, the quantity of antioxidant compounds were determined and identified by specrophotometric and HPLC chromatographic methods.

Hydrophilic antioxidants: The cellular contents of ascorbic acid (AA) and glutathione (GSH) in *Sp. plantensis* grown under H_2O_2-stress are shown in Table 1. AA and GSH contents in *Sp. plantensis* was gradually increased as response to increase of H_2O_2-stress, in dependency of time and H_2O_2 concentration. At low H_2O_2 concentration (2 mM), AA and GSH contents were increased being about 1.87 and 1.30 times that of in control cells. Whereas, under highest H_2O_2 concentration, their values were 4.3 and 2.5 fold as great as that in control cells. Therefore, H_2O_2 stress led to increased GSH biosyntheses. Compared to *Sp. plantensis* unvested, the capability of GSH level was significantly increased as resulted to increased H_2O_2 concentration in culture media. Thus, the concentration of both AA and GSH antioxidant compounds are increased as a result increasings H_2O_2 concentration and the increasing in GSH content was greater than the increase of AA. The increase in AA and GSH found in *Sp. platensis* is in accordance with results of Karpinski *et al.* [12]. They showed that GSH can act as a messenger molecule in cellular signal transduction and as a factor in plant defense against oxidative stress. These results support the hypotheses that cells of algae elevated glutathione levels in response to H_2O_2 challenge. Hence, oxidative stress enhanced the ability to synthesis GSH which induce the development of resistant of cells [36]. Noctor *et al.* [37] stated that during oxidative stress associated with catalase inhibition, or ozone exposure, GSH accumulates. High cellular GSH levels are associated to heavy metal-stress in plant cells. In other words, heavy metal exposure has been shown to lead to accelerated GSH synthesis in cultured cells [38, 39]. Similarly, GSH accumulation is found to compensate for decreases in the capacity of other antioxidants and depleted of GSH increased the sensitivity to oxidative [40]. Hence, the increasing in both of cellular AA and GSH contents are correlated with H_2O_2-stress. This may due to the role of AA in reductive detoxification of H_2O_2, which must therefore, be continuously regenerated from its oxidized form. A major function of GSH in protection against oxidative stress is re-reduction of AA in ascorbate-glutathione cycle. In this path way GSH act as a recycled intermediate in the reduction of H_2O_2 using electrons derived, ultimately from H_2O_2 [37, 41].

Lipophilic antioxidants

Tocopherol content: Tocopherols (TOH) values obtained in *Sp. plantensis* grown in media containing 2,

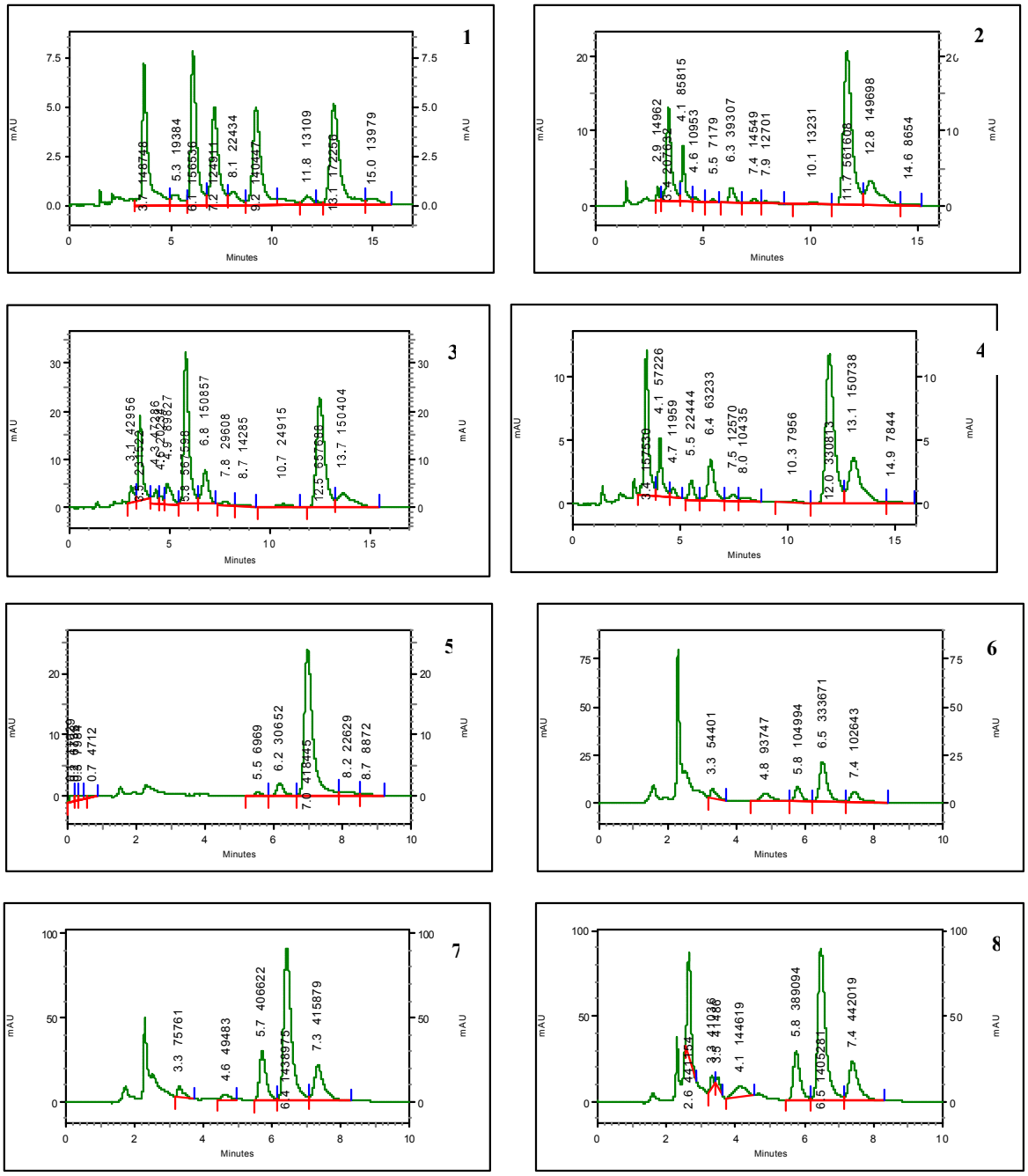

Fig. 2: HPLC profile of carotenoids and tocopherol of *Spirulina plantensis* as effected of H_2O_2 concentration

1-Standard of Carotenoids

Compound	**RT**
Astaxanthin	3.6-4.2
Lutein	5.5-6.2
Cryptoxanthine	8.9-9.5
β-carotene	12.0-13.5
Zeaxanthin	7.2-8.2

2-Sp. P. carotenoids under optimum conditions
3-Sp. P. carotenoids under 2 mM H_2O_2 / L
4-Sp. P. carotenoids under 8 mM H_2O_2 / L

5-Standard Alpha tocopherol

6-Sp. P. Tocopherol under optimum conditions
7-Sp. P. tocopherol under 2 mM H_2O_2 / L
8-Sp. P. tocopherol under 8 mM H_2O_2 / L

Table 3: Effect of hydrogen peroxide on antioxidant enzyme system sueroxide dimutase, ascobate peroxidase, catalase and peroxidase of *Spirulina plantensis*

	Ascorbate preoxidase		Preoxidase		Superoxude dismutase		Catalase	
Treatment	U mg protein^{-1}	Ratio	U mg protein^{-1}	Ratio	U mg protein^{-1}	Ratio	U mg protein^{-1}	Ratio
Control	1.35	1.00	3.17	1.0	6.22	1.00	4.12	1.00
2 mM H_2O_2/L	4.12	3.05	15.31	4.8	11.16	1.79	17.21	4.20
4 mM H_2O_2/L	6.21	4.60	18.53	5.8	19.25	3.09	25.18	6.11
6 mMH_2O_2/L	9.96	7.40	25.46	8.0	26.01	4.18	34.09	8.30
8 mM H_2O_2/L	11.64	8.62	30.46	9.6	35.62	5.72	41.32	10.03

4, 6 and 8 mM H_2O_2 were 1.69, 4.31, 5.61 and 8.36 mg g^{-1}, respectively, compared with 1.5 mg g^{-1} in control cells. In other words, cells grown under H_2O_2-stress increased the TOH content being about 1.76, 4.48, 5.84 and 8.71, respectively, times greater than in the control cells. Therefore, the increased of H_2O_2 is correlated with increased cellular TOH content. Tocopherol compounds play an important role as antioxidant, in microalgae cells and act as stabilizer of membrane lipids [42]. Tochopherol act as a chain-breaking antioxidant and as chemical scavengers of O_2 radicals, i.e., able to repair oxidizing radical directly, preventing the chain propagation step during lipid peroxidation [11, 13]. The regeneration of the oxidizing TOH to its reduced form TOH can be achieved by AA or GSH.

Total carotenoids content: The total carotenoid content of *Sp. Plantensis* (Table 2*)* was gradually increased under H_2O_2 stress in dependency on H_2O_2 concentration. The cellular content of the total carotenoids is significantly different among H_2O_2 concentration and their levels amounted to between 8.36 and 35.25 mg g^{-1}. Compared with untreated cells (4.61 mg g^{1}), the cells exposure to 2, 4, 6 and 8 mM H_2O_2 led to accumulation higher amount of total carotenoids, with values 8.36, 15.11, 22.21 and 35.25 mg g^{-1}, respectively. These values were being about 1.81, 3.28, 4.80 and 7.65 time as great as that obtained in control cells.

The HPLC chromatogram of *Sp. plantensis* carotenoids (Fig. 2) showed the presence of β-carotene, astaxanthine, luteine zeaxanthin and cryptoxanthin as the major components along with other carotenoids (unidentified peak). The identities of these carotenoids were confirmed by relative retention time and spiking with standard carotenoids. The concentrations of β-carotene, astaxanthin, luteine, zeaanthin and cryptoxanthin in control cells were 39.12, 5.61, 0.30, 1.56 and 1.69 μg g^{-1}, respectively. These values for cells grown in a media containing 2 and 8 (in parenthesis) mM H_2O_2 were 62.35 (133.54), 9.11 (26.14), 0.51 (1.32), 2.65 (5.66) and 1.69 (6.96) μg g^{-1}, respectively. On the other hand, these values increased by about 1.59 (3.41), 1.62 (4.66), 1.70 (4.38), 1.70 (3.63) and 1.4 (3.96), respectively times as that in control cells. The accumulation of carotenoids occurs when microalgae cells grown in full nutrient culture media are incubated under specific conditions that limit cell growth including nutrient imbalance (as low nitrogen levels), oxidative stress, illumination of high intensity and high concentration of salt [11, 43, 44]. The present results show that *Sp. plantensis* accumulated high amount of carotenoids when grown under H_2O_2 stress, are in accordance with previously mentioned data for *Sp. plantensis* producing carotenoids when exposed to ultraviolet β-radiation [11]. Generally, the accumulation of carotenoids in *S. plantensis* can be induced by H_2O_2 stress. The carotenoids and their derivatives act as secondary photosynthetic pigment and as protectors against chloroplastic oxygen species dissipation processes [45]. Its ability of protection against photooxidative damage has been associated to the capacity of carotenoids to dissipate the excess of light acting as filter and to their antioxidant properties [45, 46]. However, the role of carotenoids in protection of chloroplastic response against oxidative damage in one of four ways: by reacting with lipid peroxidation products to terminate the chain reaction; by scavenging singlet oxygen and dissipating the energy as heat; by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen, or by the dissipation or excess excitation energy through the xanthophylls cycle [44, 47].

Antioxidant enzymes: The activities of SOD enzyme in control and H_2O_2 exposed cells of *Sp. plantensis* are shown in Table 3. A relationship between H_2O_2 concentration and SOD activity was observed. At highest H_2O_2 concentration (8 mM) the cells exhibited the highest SOD activity with value of 35.62 U mg^{-1}

Table 4: Effect of hydrogen peroxide on free radical levels and lipid peroxidation of *Spirulina plantensis*

Treatment	Relative radical levels (%)	Lipid peroxidation MAD mM^{-1} mg protein	Ratio
Control without H_2O_2	100%	1.55	1.00
2 mM H_2O_2/L	164.2	4.32	2.78
4 mM H_2O_2 /L	250.3	6.61	4.26
6 mMH_2O_2/L	355.6	9.35	6.03
8 mM H_2O_2/L	422.3	12.61	8.14

protein, compared with 6.22 U mg^{-1} protein in control cells. However, exposed cells to H_2O_2 at 2, 4, 6 and 8 mM increased SOD activity levels with about 1.79, 3.09, 4.18 and 5.72 as great as that in untreated cells. The APX and PX activities are shown in Table 4. Under H_2O_2-stress APX and PX in algae were significantly higher ($P<0.05$) than the control. Looking at the changes in the activity of both enzymes in all treated cells, one would observe that the PX had higher activity than APX. For instance, at 2 and 8 (in parentheses) mM H_2O_2, the values of PX and APX activities were 4.12 U mg^{-1} protein (11.64 U mg^{-1} protein) and 15.31 U mg^{-1} protein (30.46 U mg^{-1} protein), respectively.

Similar trends were observed in the effect of H_2O_2-stress on CAT activity. Exposed algal to H_2O_2-stress significant correlated with the increase of CAT activity (Table 3). CAT activity was increased in cells grown at lower and highest H_2O_2 concentrations, being about 4.2 and 10.03 respectively times as great as that in control. However, H_2O_2 is produced during different metabolic processes such as photorespiration in chloroplasts [48]. H_2O_2 affects the integrity of cells because it is a precursor of highly reactive oxygen species such as hydroxyl radical ($HO^{\cdot}$) which attacks protein, lipids and nucleic acids [5]. Oxidative bursts caused by high level of H_2O_2 play a role during the defense of plant against pathogens [49]. Thus, by means of the enzyme SOD, APX, PX and CAT keep low levels of toxic compounds. However, among these enzymes CAT has the highest specific activity in U mg^{-1} protein, CAT: >1800, PX: 262-9-292; APX (34-254) [50]. The algae are more resistance to H_2O_2 than that of higher plants due to structural difference of thiol-mediated enzyme [9]. The essential for ROS detoxification during normal metabolism and particularly during stress, are antioxidant enzymes defenses system [14]. The primary scavenging enzymatic defenses system include SOD, calalase and glutathione peroxidase, (GPX) and peroxiredoxin (PrxR) [11, 15]. These enzymatic detoxification system involving the action of SOD and reductase, either quench toxic compounds or regenerate antioxidants with the help of reducing power provided by photosynthesis [16]. Our results revealed that lipophilic and hydrophilic contents in *Sp. plantensis* was positive affected by H_2O_2 concentration till the end of experiment. No or little bleaching was occurred in the *Sp. plantensis* algal (blue-color) which explain the dramatic decline in protein content due to degradation of phycobiliprotein pigment or accumulation large amount of carotenoids in cells grown under highest H_2O_2 stress. Therefore, photosynthetic efficiency was decreased in H_2O_2 stressed cell than that in control as a results of few pigments working for photosynthesis. Negative correlation between protein content and H_2O_2 stress in many algae species was found by Dummermuth *et al*. [19]. In contrast, enzyme activities of SOD, PX, APX and CAT were increased which support the data presented here for elevated enzyme activities. Finally, antioxidant compound including ascorbic acid, GSH contents in *Sp. plantensis* were increased under H_2O_2 stress. The increasing in these compounds was correlated well with an obvious stimulation of an oxidant enzyme. Treatment of plant cells with oxidants increased cell tolerance to subsequent oxidative challenges. This adaptive response could stem from the activity of antioxidant enzymes and related proteins [51].

Effect of oxidative stress on lipid peroxidation and stable free radicals: One of the expected consequences of stress-induce cellular build-up of active oxygen species is an increase in stable free radical (SFR) and lipid peroxidation. The assay of cellular accumulation lipid peroxidation products, in the form of thiobarbituric acid reactive substances as MAD and stable free radicals (measured by PER), can provide a comparative indication of such activity. Data relevant to free radicals and MAD generated in *Sp. Plantensis* cells exposed to H_2O_2 stress are shown in Table 4 and Fig. 3. The H_2O_2 stress induced progressive increases in accumulation of MAD and stable free radicals (SFR) as a biomarker for lipid peroxidation in *Sp. Plantensis* cells. The increase in their biomarker level was a gradually increased in cells grown under H_2O_2 stress in dependency of H_2O_2 concentration. The MAD values were increased in cells exposed to 2, 4, 6 and 8 mM H_2O_2 concentrations being about 2.78, 4.26, 6.03 and 8.14, respectively times as great as that found in control cells.

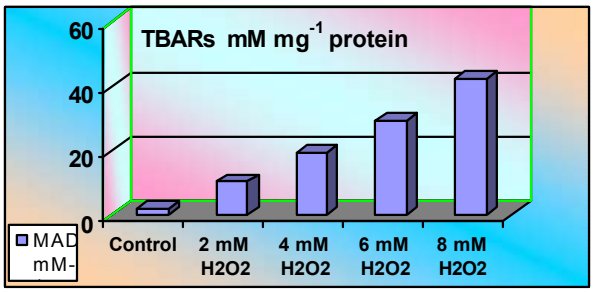

Fig. 3: Effect of hydrogen peroxide on lipid peroxidation of *Spirulina plantensis*

Figure 3 and Table 4 show the values of relative % of stable free radicals (the values recorded with g-value at maximum absorption of 2006) in lyophilized *Sp. Plantensis* cells grown under H_2O_2 stress . Treatment with 2, 4, 6 and 8 mM H_2O_2 resulted in increases relative values of SFR by nearly about 164, 250, 355 and 422% than that in control cells (100%). Therefore, increased H_2O_2 concentration in culture media enhancment the production of hydroxyl radical.OH, which leads to lipid peroxidation process. However, the enhanced of lipid peroxidation was occurred in several microalgae species may results from complexity of environmental factors such light, solar radiations and oxidative stress [11, 52].

In the photosynthetic apparatus of plants reactive oxygen species are produced mainly by excess excitation energy that lead to an over reduction of photo-system II and yield singlet-oxygen and by leakage of electrons to molecular oxygen yielding superoxide (O^{-}) electron. Consecutive reaction form H_2O_2 and the highly toxic free.OH radical [53]. Also, ROS are constantly produced in all aerobically living cells and these are potentially toxic to cells [54]. These molecules if not controlled by protective system may destroy biological molecules such pigments, lipids and proteins. The xanthophylls cycle in thylakoid membranes dissipates excess energy and helps plants to avoid the production of ROS [8]. On the other hand, carotenoids and tocopherol remove singlet oxygen directly from the pigment bed [22].

The present study shows that *Sp. Plantensis* stimulated the protective system when grown under H_2O_2 stress. The redox status of cells is maintained by an integrated net constitutive and inducible antioxidant enzyme (e.g., SOD, CAT, APX and PX) in coordination with fluctuating intercellular levels of low-molecular weight of antioxidant compounds such as GSH, AA, carotenoids and tocopherols. Algae response to stress by the increased production of antioxidant or elevated activity of protective enzymes. If the protective and repair capacities are exceeded, proteins, lipids and pigment suffer oxidation damage [11, 55]. On the other hand, algae species exhibited different tolerance and strategies against H_2O_2 exposure, which are dose and time dependent, but also, strongly correlated with antioxidant enzyme activities.

In addition to the scientific information derived from these studies, over productions of the enzymatic and non enzymatic antioxidants in *Sp. plantensis* have opened the door to novel possibilities for commercial exploitation. As a manipulatable product, GSH, AA, TOH and carotenoids have dual interest. First, its have intrinsic values as a flavors precursor and color in food and as an anti-carcinogen and antioxidant [56-58]. Due to these properties, non-enzymatic antioxidants have appreciable potential value as a medicinal products and as an additive for pharmaceutical, food or cosmetic applications. Secondly, *Sp. plantensis* can be used as a model for the biotechnological production of antioxidant compounds.

REFERENCES

1. Manley, LS., 2002. Phytogenesis of halomethanes: A product of selection or a metabolic accident. Biochem., 60: 163-180.
2. Falkowski, P.G. and J.A. Raven, 1997. Aquatic Photosynthesis. Blackwell.
3. Elstner, E.F., 1987. Metabolism of activated oxygen species. In: Davies, D.D. (Ed.). Biochemistry of Plants, Academic Press, London, 2: 253-315.
4. Blokhina, O., E. Virolainen and V.K. Fagerstedt, 2002. Antioxidants, oxidative damage and oxygen deprivation stress: A review. Ann. Bot., 91: 179-194.
5. Fridovich, I., 1978. The biology of oxygen radicals. Sci., 201: 875-880.
6. Foyer, C.H., P. Descourvieres and K.J. Kunert, 1994. Protection against oxygen radicals: Important defense mechanism studied in transgenic plants. Plant Cell Environ., 17: 507-523.
7. Apostol, I., P.F. Heinstein and P.S. Low, 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant, pp: 109-116.
8. Bischof, K., D. Hanelt and C. Wiencke, 2000. Effects of ultraviolet radiation on photosynthesis and related enzyme reactions of marine macroalgae. Planta, 211: 555-562.
9. Demming-Adams, B. and W.W. Adams, 1994. Light stress and photoprotection related to the xanthophyll cycle. In: Foyer, C. and P. Mullineaux (Eds.). Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, pp: 105-126.

10. Takeda, T., A. Yokota and S. Shigeoka 1995. Resistance of photosynthesis to hydrogen peroxide in algae. Plant Cell Physiol., 36: 1089-1095.
11. Noctor, G. and C.H. Foyer, l998. Ascorbate and glutathione: Keeping active oxygen under control. Ann. Rev. Plant Physiol. Plant Mol. Biol., 49: 249-279.
12. Abd El-Baky, H. Hanaa, F.K. El Baz and G.S. El-Baroty, 2004. Production of antioxidant by the green alga *Dunaliella salina*. Intl. J. Agric. Biol., 6(1): 49-57.
13. Karpinski, S., H. Reynolds, B. Karpinksa, G. Wingsle, G. Creissen and P. Mullineaux, 1999. Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Sci., 284: 654-657.
14. Suzuki, N. and R. Mittler, 2006. Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. Physiol. Plantarum, 126: 45-51.
15. Mittler, R., S. Vanderauwera, M. Gollery and F. Van Breusegem, 2004. Reactive oxygen gene network of plants. Trends. Plant Sci., 9: 490-498.
16. Tausz, R.M., M. Soledad and D. Grille, 1998. Antioxidative defence and photoprotection in pine needles under field conditions. A multivariate approach to evaluate patterns of physiological responses at natural sites. Physiol. Planta., 104: 760-768.
17. Alscher, R.G., J.L. Donahue and C.L. Cramer, 1997. Reactive oxygen species and antioxidants: Relationships in green cells. Physiol. Plant, 100: 224-233.
18. Fadzillah, N.M., V. Gill, R.P. Finch and R.H. Burdon, 1996. Chilling, oxidative stress and antioxidant responses in shoot cultures of rice. Planta, 199: 552-556.
19. Dummermuth, L.A., U. Karsten, K.M. Fisch, G.M. Kónig and C. Wiencke, 2003. Responses of marine macroalgae to hydrogen-peroxide stress. J. Experim. Marine Biol. Ecol., 289: 103-121.
20. Abd El-Baky, H. Hanaa, F.K. El Baz and G.S. El-Baroty, 2003. *Spirulina* species as a source of carotenoids and α-tocopherol and its anticarcinoma factors. Biotechol., 2(3): 222-240.
21. Prasad, K.V.S.K., P.P. Saradhi and P. Sharmila, 1999. Concerted action of antioxidant enzymes and curtailed growth under zinc toxicity in *Brassica juncea*. Environ. Exp. Bot., 42: 1-10.
22. Polle, A. and H. Rennenberg, 1994. Photooxidative stress in trees. In: Foyer, C.H. and P.M. Mullineaux (Eds.). Causes of photoxidative stress and amelioration of defense Systems in Plants. Boca Raton, FL: CRC Press.
23. Foyer, C.H. and G. Noctor, 2000. Oxygen processing in photosynthesis: Regulation and signaling. Rev. New Phytol., 146: 359-388.
24. Zarrouk, C., 1966. Contribution a l'etude d; une cyanophycee. Influene de divers facteurs physiques et chimiques sur la croissance et la photosynthese de *Spirulina maxima* (Setch. et Gardner) Geitler. Ph.D. thesis, University of Paris, France.
25. Payer, H.D., 1971. First report upon the organization and experimental work of the Thailand German project on the production and utilization of single cell green algae as a protein source for human nutrition. Inst. Food Res. Product Development Kasetsar Univ., Bangkok, Thailand.
26. Semenenko, E.V. and A.A. Abdullaev, 1980. Parametric control of β-carotene biosynthesis in *Dunaliella salina* cells under conditions of intensive cultivation . Fizioloiya, Rastenii., 27: 31-41.
27. Honya, M.K., T. Kinoshita, M. Ishikawa, H. Mori and K. Nisizw, 1994. Seasonal variation in lipid content of cultured *Laminaria japonica* fatty acids,srerols, β-carotene and tocopherol. J. Appl. Phycol., 6: 25-29.
28. Augustin, J., P.B. Klein, D. Becker and B.P. Venugopal, 1985. Vitamin In: Methods of Vitamin Assay. Academic Press, Now York, USA, pp: 323.
29. Ginnopolitis, N.C. and S.K. Ries, 1977. Superoxide dismutase occurrence in higher plants. Plant Phys., 59: 309-314.
30. Chance, B. and A.C. Maehly, 1955. Assay of catalase and peroxidase. In: Colowic, S.P.and N.O. Kaplan (Eds.). Methods of Enzymology, Academic Press, New York, USA, 2: 764.
31. Hans-Luck, 1970. Catalase. In: Verlag Chemie Gmb H. (Ed.). Method of Enzymatic Analysis. Weinheim Bergstr, Academic Press, New York and London, pp: 885-888.
32. Nakano, M. and K. Asada, 1981. Hydrogen Peroxide is scavenged by ascorbate-specific peroxidase in Spinach Chloroplasts. Plant Cell Physiol., 22: 867-880.
33. Silber, R., M. Farber, E. Papopoulos, D. Nervla, L. Liebes, M. Bruch and R. Bron, 1992. Glutathione depletion in chronic lymphocytic leukemia B-lymphocytes. Blood, 80: 2038-2040.
34. Haraguchi, H., H. Ishikawa and I. Kubo, 1997. Antioxdative action of di-terpenoids from *Podocarpus nagi*. Planta Medica, 63: 213-215.
35. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram of protein utilizing of protein-dye binding. Anal. Biochem., 72: 248-258.

36. Ridnour, A.L., E.J. Sim, J. Choi, A.D. Dickinson, H.J. Forman, M.I. Ahmad, C.M. Coleman, R.C. Hunt and R.D. Spitz, 2005. Nitric oxide-induced resistance to hydrogen peroxide stress is a glutamate cysteine ligase activity-dependent process. Free Rad. Biol. Med., 38: 1361-1371.
37. Noctor, G., A.M. Aris, J. Jouanin J.K. Kunert, H. Rennenberg and C.H. Foyer 1998. Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J. Exp. Bot., 321: 623-647.
38. Luwe, M., 1996. Antioxidants in the apoplast and symplast of beech (*Fagus sylvatica L.*) leaves: Seasonal variations and responses to changing ozone concentrations in air. Plant, Cell and Environ., 19: 321-328.
39. Schneider, S. and L. Bergmann, 1995. Regulation of glutathione synthesis in suspension cultures of parsley and tobacco. Botanica Acta, 108: 34-40.
40. Grant, C.M., F.H. MacIver and I.W. Dawes, 1996. Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. Current Gene., 29: 511-515.
41. Foyer, C.H., 1997. Oxygen metabolism and electron transport in photosynthesis. In: Scandalios, J.G. (Ed.). Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbor, New York, USA, Cold Spring Harbor Laboratory, pp: 587-621.
42. Giansuddin, A.S.,M. and A.T. Diplock, 1981. The influence of vitamine E on membrane lipids of mouse fibroblast in culture. Arch. Biochem. Biophys., 210: 348-362.
43. El-Baz, F.K., A.M. Aboul-Enein, G.S. El-Baroty, A.M Youssef and Abd El-Baky, H. Hanaa, 2002. Accumulation of antioxidant vitamins in *Dunaliella salina*. Online J. Biolog. Sci., 2 (4): 220-223.
44. Salguero, A., M. Benito, J. Vigara, J.M. Vega, C. Vilchez and R. León, 2003. Carotenoids as protective response against oxidative damage in *Dunaliella bardawil* Biomol. Eng., 20: 249-253.
45. Bennoun, P., 1998. In: Rochais, J.D., M. Goldschmidt, S. Merchant (Eds.). The molecular biology of chloroplasts and mitochondria in *Chlamydompnas*. Dordrecht: Kluwer Academic Publisher, pp: 675-683.
46. Ben-Amotz, A. and J. Shaish, 1992. In: Ben-Amotz, A. and M. Averon, (Eds.). *Dunaliella:* physiology, biochemistry and biotechnology, USA, CRC Press, pp: 135-164.
47. Osmond, B., M. Badger, K. Maxwell, O. Björkman and R. Leegod, 1997. Too many photons: Photorespiration, photoinhibition and photooxidation. Trends Plant Sci., 2: 119-120.
48. Henzler, T. and E. Steudle, 2000. Transport and metabolic degradation of hydrogen peroxide in *Chara coralline*: Model calculations and measurements with the pressure probe suggest transport of H_2O_2 water channels. J. Exp. Bot., 51: 2053-2066.
49. Peng, M. and J. Kuc, 1992. Peroxidase-generated hydrogen peroxide as a source of antifungal activity *in vitro* and on tobacco leaf disks. Phytopathol., 82: 696-699.
50. Schomburg, D., M. Salzmann and D. Stephan, 1994. Enzyme handbook 7. Berlin, Heidelberg: Springer-Verlag.
51. Malanga, G., G. Calmanovici and S. Puntarulo, 1997. Oxidative damage to chloroplasts from *Chlorella vulgaris* exposed to Ultraviolet B radiation. Physiol. Plant., 101: 455-462.
52. Yamasaki, H. and C.S. Grace, 1998. EPR detection of phytophenoxyl radicals stabilized by zinc ions: Evidence for the redox coupling of plant phenolics with ascorbate in the H_2O_2-peroxidase system. FEBS Letters, 422: 377-380.
53. Elstner, E.F. and W. Osswald, 1994. Mechanisms of oxygen activation during plant stress. Proc. R. Soc. Edinburgh, 102B: 131-154.
54. Rao, M.V., G. Paliyath and D.P. Ormrod, 1996. Ultraviolet-B-and ozone induced biochemical changes in antioxidant enzymes of Arabidopsis thaliana. Plant Physio1., 110: 125-136.
55. Barros, P.M., M. Granbom, P. Colepicolo and M. Pedersen, 2003. Temporal mismatch between induction of superoxide dismutase an d ascorbate peroxidase correlates with high H_2O_2 concentration in seawater from clofibrate-treated red algae *Kappaphycus alvarezii*. Arch. Biochem. Biophys., 420: 161-168.
56. Ho, C.T., Y.C. Oh, Y. Zhang and C.K. Shu, 1992. Peptides as flavor precursors in model Maillard reactions. ACS symposium series. Washington DC: American Chemical Society, pp: 193-203.
57. Jones, D.P., R.J. Coates, E.W. Flagg, J.W. Eley, G.H. Block, R.S. Greenberg, E.W. Gunter and B. Jackson, 1992. Glutathione in foods listed in the national cancer institutes health habits and history food frequency questionnaire. Nut. Cancer, 17: 57-75.
58. Abd El-Baky and H. Hanaa, 2003. Over production of Phycocayanin pigment in blue green alga *Spirulina Sp.* and its inhibitory effect on growth of Ehrlich ascites carcinoma cells. J. Med. Sci., 3 (4): 314-324.